IN VITRO ANTI-CANCER ACTIVITIES OF *VITEX NEGUNDO* AND *HELIOTROPIUM INDICUM* EXTRACTS AGAINST HUMAN CANCER CELL LINES

A Dissertation submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY,

CHENNAI- 600 032

In partial fulfilment of the award of the degree of

MASTER OF PHARMACY

IN

Branch- IV - PHARMACOLOGY

Submitted by

Name: JENET JEMILAMARY.V.A

REG.No.261425222

Under the Guidance of

Dr. A. Prakash, M. Pharm., Ph. D

DEPARTMENT OF PHARMACOLOGY

J.K.K. NATTARAJA COLLEGE OF PHARMACY

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TAMILNADU.

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CERTIFICATES

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"*In vitro* anti-cancer activities of *Vitex negundo* and *Heliotropium indicum* extracts against human cancer cell lines "** submitted by the student bearing **Register no: 261425222** to **"The Tamil Nadu Dr M. G. R. Medical University – Chennai"**, in partial fulfilment for the award of Degree of **Master of Pharmacy in Pharmacology** is the bonafide work carried out under the guidance and direct supervision of **Dr. A. PRAKASH**, Department of Pharmacology and was evaluated by us during the examination held on……...

INTERNAL EXAMINER **EXTERNAL EXAMINER**

CERTIFICATE

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Dr. A. Prakash, M.Pharm, Ph.D
Guide

Dr. R. Shanmugasundaram, M.Pharm., Ph.D
HOD

Dr. R. Sambath Kumar, M.Pharm., Ph.D
Principal

CERTIFICATE

This is to certify that the dissertation work entitled **"*In vitro* anti-cancer activities of *Vitex negundo* and *Heliotropium indicum* extracts against human cancer cell lines "** submitted by the student bearing **Register no: 261425222** to **"The Tamil Nadu Dr M. G. R. Medical University – Chennai",** in partial fulfilment for the award of Degree of **Master of Pharmacy in Pharmacology** is the bonafide work carried out under my guidance and direct supervision in the Department of Pharmacology, J.K.K. Nattraja College of Pharmacy, Kumarapalayam, during the academic year 2015-16.

Place: Kumarapalayam
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DECLARATION

I do hereby declare that the dissertation work entitled **"*In vitro* anti-cancer activities of *Vitex negundo* and *Heliotropium indicum* extracts against human cancer cell lines "** submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the award of Degree of **Master of Pharmacy** in **Pharmacology**, was done under the guidance of **Dr. A. PRAKASH., M. Pharm., Ph. D** at the Department of Pharmacology, JKK Nattraja College of Pharmacy, Kumarapalayam, during the academic year 2015-2016.

I further declare that this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associate ship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

Place: Kumarapalayam **JENET JEMILAMARY.V.A**

Date: **Reg. No: 261425222**

ACKNOWLEDGEMENT

I am proud to dedicate my deep sense of gratitude to the founder, (Late) Thiru **J.K.K. Nattaraja Chettiar**, providing us the historical institution to study.

My sincere thanks and respectful regards to our reverent Chairperson **Smt. N. Sendamaraai, B.Com**., and the Managing Director **Mr. S. Omm Sharravana, B.Com., LLB.,** J.K.K. Nattraja Educational Institutions, Kumarapalayam for their blessings, encouragement and support at all times.

It is most pleasant duty to thank for our beloved Principal **Dr. R. Sambath kumar, M. Pharm., Ph.D**, J.K.K. Nattraja College of Pharmacy, Komarapalayam for ensuring all the facilities were made available to me for the smooth running of this project.

Our glorious acknowledgement to our administrative officer **Dr. K. Sengodan, M.B.B.S.,** for encouraging us in a kind and generous manner to complete this work.

I express whole hearted gratitude to my guide **Dr. A. Prakash., M. Pharm., Ph. D** Assistant Professor, Department of Pharmacology, for suggesting solution to problems faced by me and providing indispensable guidance, tremendous encouragement at each and every step of this dissertation work. Without his critical advice and deep-rooted knowledge, this work would not have been a reality.

My sincere thanks to **Dr. R. Shanmuga Sundaram, M. Pharm., Ph.D., Vice Principal, Professor & Head,** Department of Pharmacology**, Mrs. Baby Kala M.Pharm.,** Lecturer, Department of Pharmacology, **Mrs. R. Elavarasi, M.Pharm.,** Lecturer, Department of pharmacology and **Mrs. Vannamalar, M.Pharm**., Lecturer, Department of Pharmacology.

My sincere thanks to **Mrs. S. Bhama, M. Pharm.,** Assistant Professor Department of Pharmaceutics, **Mr. R. Kanagasabai, B.Pharm, M.Tech.,** Assistant Professor, **Mr. K.**

Jaganathan, M.Pharm., Assistant Professor, Department of Pharmaceutics, **Mr. C. Kannan M.Pharm.,** Assistant Professor, Department of Pharmaceutics and **Mr. V. Kamalakannan M.Pharm.,** Assistant Professor, Department of Pharmaceutics for their valuable help during my project.

My sincere thanks to **Mr. N. Venkateswaramurthy, M.Pharm., Ph.D.,** Professor and Head, Department of Pharmacy Practice. **Dr. Radhika, M.Pharm., Ph.D.,** AssociateProfessor, **Dr. K. Sattanathan, M.Pharm., Ph.D.,** Assistant Professor **Mrs. K. KrishnaVeni, M.Pharm.,** Lecturer, Department of Pharmacy Practice, **Mrs. P. Kavitha M.Pharm** Lecturer, Department of Pharmacy Practice, **Mr. R. Kameswaran M.Pharm** Lecturer Department of Pharmacy Practice, **Dr.Taniya Jacob, Pharm.D.,** Lecturer, Department of Pharmacy Practice, **Dr. V. Viji Queen, Pharm.D.,** Lecturer, Department of Pharmacy Practice, **Mrs. M. Sudha M.Pharm.,** Lecturer, Department of Pharmacy Practice and **Mr. T. Thiyagarajan M.Pharm** Lecturer Department of Pharmacy Practice, for their help during my project.

It is my privilege to express deepest sense of gratitude toward **Dr. M. Vijayabaskaran**, **M.Pharm.,** Professor & Head, Department of Pharmaceutical chemistry, **Dr. S. P. Vinoth Kumar M.Pharm.,** Assistant professor, Department of Pharmaceutical chemistry, **Mrs. S. Gomathi M.Pharm.,** Lecturer, Department of Pharmaceutical chemistry, **Mrs. B. Vasuki M.Pharm.,** Lecturer, Department of Pharmaceutical chemistry, for their valuable suggestions and inspiration.

My sincere thanks to **Dr. V. Sekar, M.Pharm., Ph.D.,** Professor and Head, Department of Analysis, **Mr. S. Jayaseelan, M.Pharm.,** Associate Professor, **Dr. I. CaolinNimila, M.Pharm., Ph.D.,** Assistant Professor, and **Mr. K.K. Senthil Kumar**, **M.Pharm.,** Assistant Professor, Department of Pharmaceutical Analysis for their valuable suggestions.

My sincere thanks to **Dr. Senthilraja, M.Pharm., Ph.D.,** Professor and Head, Department of Pharmacognosy, **Dr. Rajkumar, M.Pharm., Ph.D.,** Professor**,** Department of Pharmacognosy**, Mrs. Meena Prabha M.Pharm.,** Lecturer, Department of Pharmacognosy and **Mrs. Seema, M.Pharm.,** Lecturer, Department of Pharmacognosy for their valuable suggestions during my project work.

I greatly acknowledge the help rendered by **Mrs. K. Rani,** Office Superintendent, Miss**. Venkateswari, M.C.A.,** typist**, Mrs. V.Gandhimathi, M.A., M.L.I.S.,** Librarian, Mrs**. S. Jayakala B.A., B.L.I.S.,** and Asst. Librarian.

I owe my thanks to all the technical and non-technical staff members of the institute for their precious assistance and help.

Last, but nevertheless, I am thankful to my colleagues and all my friends for their co-operation, encouragement and help extended to me throughout my project work.

JENET JEMILAMARY.V.A

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INTRODUCTION

1. INTRODUCTION

Many of today's synthetic drugs originated from the plant kingdom, and only about 200 years ago our pharmacopoeia was dominated by herbal medicines [1]. The largest research fields, as defined by the number of publications describing bioactive plant-derived compounds in the last few years, are anti-tumour drugs, antibiotics, drugs active against tropical diseases, contraceptive drugs, anti-inflammatory drugs, immunomodulators, kidney protectors and drugs for psychiatric use [2]. Herbal drugs are being proved as effective as synthetic drugs with lesser side effects [3].

Plants have a great potential for producing new drugs for human benefit. Plants used in traditional medicine contain a vast array of substances that can be used to treat chronic and even infectious diseases. According to a report of World Health Organization, more than 80% of world's populations depend on traditional medicine for their primary health care needs [4].

WHO encourages countries to provide safe and effective traditional remedies and practices in public and private health services and it also published two monographs on medicinal plants with information on pharmacopoeial summaries for quality assurance: botanical features, distribution, identity tests, purity requirements, chemical assays, and active or major chemical constituents, clinical applications, pharmacology, contraindications, warnings, precautions, potential adverse reactions, and posology, etc.

The Indian flora is extensively utilized as source of many drugs mentioned in the traditional systems of medicine. During the last few decades there has been an increasing interest in the study of medicinal plants and their traditional use in different parts of India. Indian medicinal plants are widely used by all sections of the population and it has

been estimated that over 7500 species of plants are used by several ethnic communities. India possesses more than 500 tribal communities and even today, tribals and certain local communities in India practice herbal medicine to cure a variety of diseases and disorders [5].

The demand for more and more drugs from plant sources is continuously increasing. It is therefore essential for systematic evaluation of plants used in traditional medicine for various ailments. The increased interest in plant derived drugs is mainly because of the wide spread belief that 'herbal medicine' is safer than costly synthetic drugs which possesses side effects [6]. Drug discovery from medicinal plants continues to provide new and important leads against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, and pain. Hence, there is need to screen medicinal plants for promising biological activity.

Cancer is a major public health burden in both developed and developing countries. Cancer is the second leading cause of death in the United States [7], where one in four deaths is due to cancer. Plants have long been used in the treatment of cancer [8]. The National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity [9]. Of the 92 anticancer drugs commercially available prior to 1983 in the US and among worldwide approved anticancer drugs between 1983 and 1994, 60% are of natural origin [10].

1.1 Plant derived anticancer agents in clinical use

The isolation of the vinca alkaloids, vinblastine and vincristine from the Madagascar periwinkle, *Catharanthus roseus* (Apocynaceae) introduced a new era of the use of plant material as anticancer agents. They were the first agents to advance into

clinical use for the treatment of cancer [11]. Vinblastine and vincristine are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma [11]. The discovery of paclitaxel (Taxol®, **3**) from the bark of the Pacific Yew, *Taxus brevifolia* (Taxaceae), is another evidence of the success in natural product drug discovery [12]. Numerous types of bioactive compounds have been isolated from plant sources. Several of them are currently in clinical trials or preclinical trials or undergoing further investigation.

1.2 Burden of Cancer in India

It is estimated that there are approximately 2 - 2.5 million cases of cancer in the country at any given time. Nearly 800,000 cases were diagnosed in the year 2000 and 550,000 deaths due to cancer occurred in the Indian population. The tobacco related cancers account for almost a third of cancers diagnosed in head and neck, lung and oesophagus in the Indian population. The two most common cancers of women viz. cancer of the cervix and breast, further account for half the cancer burden in Indian women [13].

1.3 History of cancer

Today, the Greek term carcinoma is the medical term for a malignant tumor derived from epithelial cells. It is Celsus who translated *carcinos* into the Latin *cancer*, also meaning crab. Galen used "*oncos*" to describe *all* tumours, the root for the modern word oncology [13].

Hippocrates described several kinds of cancers. He called benign tumours *oncos*, Greek for swelling, and malignant tumours *carcinos*, Greek for crab or crayfish. This

name probably comes from the appearance of the cut surface of a solid malignant tumour, with a roundish hard center surrounded by pointy projections, vaguely resembling the shape of a crab. He later added the suffix *-oma*, Greek for swelling, giving the name *carcinoma* [13-15]. Since it was against Greek tradition to open the body, Hippocrates only described and made drawings of outwardly visible tumors on the skin, nose, and breasts. Treatment was based on the humor theory of four bodily fluids (black and yellow bile, blood, and phlegm). According to the patient's humor, treatment consisted of diet, blood-letting, and/or laxatives. Through the centuries it was discovered that cancer could occur anywhere in the body, but humor-theory based treatment remained popular until the 19th century with the discovery of cells.

The first known surgical treatment for cancer was described in the 1020s by Avicenna (Ibn Sina) in *The Canon of Medicine*. He stated that the excision should be radical and that all diseased tissue should be removed, which included the use of amputation or the removal of veins running in the direction of the tumor. In the 16^{th} and 17^{th} centuries, it became more acceptable for doctors to dissect bodies to discover the cause of death [13-16]. The German professor Wilhelm Fabry believed that breast cancer was caused by a milk clot in a mammillary duct. The Dutch professor Francois de la Boe Sylvius, a follower of Descartes, believed that all disease was the outcome of chemical processes, and that acidic lymph fluid was the cause of cancer. His contemporary Nicolaes Tulp believed that cancer was a poison that slowly spreads, and concluded that it was contagious.

With the widespread use of the microscope in the 18^{th} century, it was discovered that the 'cancer poison' spread from the primary tumor through the lymph nodes to other

sites ("metastasis"). This view of the disease was first formulated by the English surgeon Campbell De Morgan between 1871 and 1874. The use of surgery to treat cancer had poor results due to problems with hygiene. The renowned Scottish surgeon Alexander Monro saw only 2 breast tumor patients out of 60 surviving surgery for two years. In the 19th century, asepsis improved surgical hygiene and as the survival statistics went up, surgical removal of the tumor became the primary treatment for cancer. With the exception of William Coley who in the late 1800s felt that the rate of cure after surgery had been higher *before* asepsis (and who injected bacteria into tumors with mixed results), cancer treatment became dependent on the individual art of the surgeon at removing a tumor. During the same period, the idea that the body was made up of various tissues, that in turn were made up of millions of cells, laid rest the humor-theories about chemical imbalances in the body. The age of cellular pathology was born [13-17].

When Marie Curie and Pierre Curie discovered radiation at the end of the 19th century, they stumbled upon the first effective non-surgical cancer treatment. With radiation came also the first signs of multi-disciplinary approaches to cancer treatment. The surgeon was no longer operating in isolation, but worked together with hospital radiologists to help patients. The complications brought, along with the necessity of the patient's treatment in a hospital facility rather than at home, also created a parallel process of compiling patient data into hospital files, which in turn led to the first statistical patient studies.

Cancer patient treatment and studies were restricted to individual physicians' practices until World War II, when medical research centers discovered that there were large international differences in disease incidence. This insight drove national public

health bodies to make it possible to compile health data across practises and hospitals, a process that many countries do today. The Japanese medical community observed that the bone marrow of bomb victims in Hiroshima and Nagasaki was completely destroyed. They concluded that diseased bone marrow could also be destroyed with radiation, and this led to the discovery of bone marrow transplants for leukemia. Since WWII, trends in cancer treatment are to improve on a micro-level the existing treatment methods, standardize them, and globalize them as a way to find cures through epidemiology and international partnerships.

1.4 Cancer

Cancer is large and complex family of malignancies manifested with uncontrolled and undifferentiated cellular growth that can affect virtually every organ in the body. Cancer begins in the body cells, which are constantly dividing and multiplying to replace old and damaged cells. Sometimes, cells begin to divide unnecessarily, forming excess tissue known as tumor.

1.4.1 Types of Cancer [17]

There are many different types of cancer. They fall into four broad categories.

- **Carcinomas**

  Carcinomas are the tumors that arise in the surface lining of tissues of visceral organs. About 80% of all cancer cases are carcinomas.

- **Sarcoma**

  Sarcomas are the tumors that originate in bone, muscles, cartilage, fibrous tissue or fat.

❖ **Leukemia**

Leukemia is a cancer of the blood or blood forming organs.

❖ **Lymphoma**

Lymphomas affect the lymphatic system, a network of vessels and nodes that acts as the body's filter. The lymphatic system distributes nutrients to blood, tissue and prevents bacteria and other foreign invaders from entering the bloodstream. There are over 20 types of lymphoma.

Carcinogens, the agents that cause cancer, have been classified into three broad groups' *viz.*: physical, chemical and biological.

✓ **Physical Agents**:

- Ultraviolet and ionizing radiations (X-rays, γ-rays).

✓ **Chemical Agents**:

Chemical carcinogens can be classified as:-

a) Initiating agent – which is capable of initiating cells only.

b) Promoting agent – capable of causing the expression of initiated cell clones.

c) Progressor agent – which can convert initiated cell or a cell in the stage of promotion to a potentially malignant cell.

A complete carcinogen has all the properties of initiating, promoting and progressor agents.

✓ **Biological Agents**

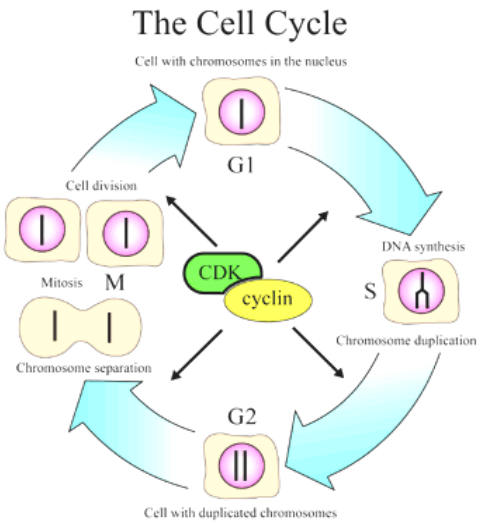
The oncogenic viruses (RNA viruses, the retroviruses) are well known and form a very diverse group of carcinogenic agents.

1.5 Cell cycle inhibition [15-17]

The cell cycle refers to the process of cell division. In normal tissues this process is tightly controlled by the activity of a number of key cellular proteins. A loss of cell cycle control may lead to an inappropriate proliferation of cells and ultimately leads to tumor formation. The cell cycle consists of:

- G_1 = growth and preparation of the chromosomes for replication
- S = synthesis of DNA (and centrosomes)
- G_2 = preparation for chromosome duplication
- M = mitosis

Abnormalities in the cell cycle of cancer cells may lead to abnormal proliferation and/or a failure to respond appropriately to DNA damage (repair or apoptosis).



The cell cycle checkpoints are regulated by protein complexes consisting of cyclins, cyclin dependent kinases and their inhibitors.

1.5. 1 Cells die by two primary processes

a. Necrosis

Necrosis is a process in which the release of intracellular proteases and lysozymes induce an inflaµMatory response. Cell death by necrosis usually follows major pathological acute injury such as hypoxia, hyperthermia, and viral invasion, exposure to various exogenous toxins or attack by complement. Early mitochondrial swelling, dysfunction of the plasma membrane with loss of homeostasis.

b. apoptosis

Apoptosis or programmed cell death plays a major role during development, homeostasis and immune response in multicellular organism. The inappropriate initiation of apoptosis is thought to contribute to the etiology of many diseases, including neurodegenerative disorders and autoimmune diseases, including type-1 diabetes. Conversely, with this a failure to undergo apoptosis (inappropriate cell survival) and proliferation can also lead to pathologies, such as tumor development and cancer. Likewise, several reports identify apoptosis cell death as an important cellular mechanism to be utilized in the treatment of tumors. A greater understanding of the control of apoptosis may allow prophylactic or therapeutic modulation of the process to prevent or protect from the diseases mentioned.

The effectors of apoptosis

There is a hierarchical organization to the pathway of apoptosis. This includes

a. The intrinsic pathway of apoptosis.
b. The extrinsic pathway of apoptosis.

1.6 Chemotherapy [15-17]

Chemotheraphy is the treatment of cancer with drugs ("anticancer drugs") that can destroy cancer cells. In current usage, the term "chemotherapy" usually refers to *cytotoxic* drugs which affect rapidly dividing cells in general.

Chemotherapy refers to the use of medication and drugs for treatment of cancer. Most chemotherapeutic drugs are cytotoxic and they work by killing cells. They do this by preventing the formation of new DNA or by blocking some other essential function in the cell. There is a limit on how much of these drugs could be safely used. Since all the chemotherapy drugs interfere with growth of the cells, they all have some form of undesired side effects. The maximum tolerated dose of a drug is determined by the profile of its side effects. These drugs can still be used safely, as long as damage is reversible and not life-threatening. Scientists took this concept to a different phase. They allowed the side effects to occur, but somehow overcame the life-threatening side effects. Chemotherapeutic drugs act in a cell cycle specific manner, that is their action predominates in specific phase of the cell cycle.

Cytotoxicity Studies

Tissue culture has been used to screen many anticancer drugs since there is clear correlation between the *in vitro* and *in vivo* activities of potential chemotherapeutic agents. There is scientific justification for cytotoxicity testing in tissue culture, since animal models are in many ways inadequate for predicting the effects of chemicals on humans since there are many metabolic differences between species. Cytotoxicity studies involve the analysis of morphological damage or inhibition of zone of outgrowth induced by the chemicals tested.

REVIEW OF LITERATURE

2 LITERATURE STUDIES

2. 1 *Vitex negundo*

Geographical distribution: *Vitex* usually grows from three to nine feet tall, but under cultivation can develop to 20 feet tall. Nirgundi occur in tropical to temperate regions (up to 2200 m from east to west) grows gregariously in wastelands and is also widely used as a hedge-plant. This species is globally distributed in Indo-Malasia, cultivated in America, Europe, Asia and West Indies. Within India, it is found throughout the greater part of India, ascending to an altitude of 1500 meter in the outer Himalayas. It is abundant in open-waste lands. Locally distributed throughout the State Maharashtra along the banks of rivers; very common near the sea-coastal and beach-forests in Konkan; along Deccan rivers. Habitat found to be in Waste lands and moist situations. A small slender tree with quadrangular branchlets densely whitish, tomentose branchlets distributed throughout India. It is often found growing next to streams and it loves water [18-20].

Cultivation: It is widely planted as a hedge plant in between the fields and usually not browsed by the cattle. It can be reproduced readily from shoot cuttings. It produces root suckers which can also be utilized as planting material. An easily grown plant, it prefers a light well-drained loamy soil in a warm sunny position sheltered from cold drying winds succeeds in poor dry soils. Plants tolerate temperatures down to about -10°C. The leaves and stems are strongly aromatic. The flowers have a most pronounced musk-like perfume.

Leaves: The leaves of *Vitex negundo* Linn are used as antibacterial, antitumor, astringent, febrifuge, sedative, tonic and vermifuge. They are useful in dispersing swellings of the joints from acute rheumatism and of the testes from suppressed gonorrhea. The juice of

the leaves is used for removing foetid discharges and worms from ulcers, whilst oil prepared with the leaf juice is applied to sinuses and scrofulous sores. Extracts of the leaves have shown bactericidal and antitumor activity. Leaves are antiparasitical, alterative, aromatic, vermifuge, pain reliever. Leaves are insect repellents. Extracts of the leaves have insecticidal activity. The fresh leaves are burnt with grass as a fumigant against mosquitoes. Decoction of leaves may improve eyesight [21-23].

Dosage: Nirgundi Juice - 20 to 30 ml per day. Nirgundi leaf Powder - 3 to 6 grams per day.

Stem: A decoction of the stems of *Vitex negundo* Linn is used in the treatment of burns and scalds.

Fruit: The dried fruit of *Vitex negundo* Linn is used as vermifuge. The fruit is also used in the treatment of angina, colds, coughs, rheumatic difficulties etc. The fresh berries are pounded to a pulp and used in the form of a tincture for the relief of paralysis, pains in the limbs, weakness etc. Fruit-nervine, cephalic, emengogue, dried fruit-vermifuge employing an aqueous extract from the fruit, a 1979 study reported good results on premenstrual water retention. Women were able to sustain a good level of milk production for breast feeding while taking this herb. While it took some time for the drug to take effect, the women were able to continue the use of the drug for months without harmful side effects [20].

Root: The root of *Vitex negundo* Linn is expectorant, febrifuge and tonic. It is used in the treatment of colds and rheumatic ailments. It is harvested in late summer and autumn and dried for later use. Roots are tonic, febrifuge, and expectorant, diuretic. Root juice is said to increases the growth of hair.

Seed: Seeds of *Vitex negundo* Linn. Occasionally used as a condiment, it has pepper substitute. When washed to remove the bitterness it can be ground into a powder and used as flour.

Phytochemical studies of *Vitex negundo* have afforded several types of compounds, such as volatile oils, lignans, flavonoids, terpenes (triterpenes, diterpenes, sesquiterpenes) and steroids [24].

The most common flavonoid glycosides from an ethanolic extract of the leaves of *Vitex negundo* are 5-hydroxy-3, 6, 7-trimethoxy-2-(3, 4-dimtoxypheny) -4H- chrome -4-on and 5, 7-dihydroxy-2-(3, 4-dihydroxyphenyl)-4H-chromen- 4-one. The methanolic extract also contains, Negundoside, Agnuside, and Vitegnoside. From bark of *Vitex negundo* Linn, *p*-hydroxybenzoic acid and β-sitosterol have been isolated, and identified from the methanol and hexane extracts of *Vitex negundo*.

In the acetoacetate fraction of the seeds , two phenylnaphtha-lene-typelignans have been- obtained and identified as 6-hydroxy-4-(4-hydroxy-3-methoxy-phenyl)-3-hydroxy-methyl-7-methoxy-3, 4-dihydro-2-naphthaldehyde and vitedoamine A. It is used to treat dyspepsia, colic, rheumatism, worms, boils and leprosy. The roots contain a furanoeremophilane. Tyrosinase inhibitory lignins have been found in the methanol extract of the roots of *Vitex negundo* [25].

Aranda *et al* [26] studied the extract of *Vitex negundo* L. was prepared by percolation method. *Vitex negundo* L. showed significant ($p<0.05$) reduction in DAI, macroscopic and microscopic lesion score as well as significant ($p<0.05$) improvement in MPO, MDA, CAT, and SOD level as compared to Group B. The ethanolic extract of

leaves of *Vitex negundo* L. showed significant amelioration of experimentally induced colitis, which may be attributed to its anti-inflammatory and antioxidant property.

2.2 *Heliotropium indicum*

Heliotropium indicum (Boraginaceae) commonly called ‘Hatisura’ in Hindi is distributed widely throughout India. The name "heliotrope" originates from the old idea that the inflorescence of these plants turned their rows of flowers to the sun. The meaning of ‘helios’ in Greek is ‘sun’ and ‘tropein’ from where the word ‘tropium’ is derived means ‘to turn’ [27]

Heliotropium indicum belongs to family Boraginaceae. This family is well marked in their characteristics and not easily confused with any other. A majority of the plants in the family are herbs. Several heliotropes are popular garden plants and some others occur as weeds.

The genus *Heliotropium* comprises about 250 species and is distributed in tropical, subtropical and warm temperate zones of all continents, but only a few species have been systematically investigated.

Heliotropium indicum commonly known as ‘Indian heliotrope’ is very common in India and some parts of Africa and Bangladesh, but also found in other countries. It is a coarse foetid herb, up to 2 ft. high, with ascending hirsuit branches found throughout India in sunny localities, on waste lands and anthropogenic habitats in periodically desiccating pools and ditches and anthropogenic habitats, generally below 800 m altitude, widely considered as a weed of fields. The leaves are simple, alternate or sub-opposite, 4.5 to 10 cm/2.5 to 5 cm, ovate or ovate oblong, margin undulate, sparsely strigose along nerves on either side, serulate or undulate with cordate, minutely pilose beneath nerves

and veins conspicuous on the lower side *H. indicum* may flower throughout the year; the flowering season is very long and new flowers develop apically within the cyme while mature nutlets are already present at the base of the inflorescence. The flowers are white or violet coloured, regular, sessile, two ranked pentamerous, extra axillary. Sepals-5, 2.5 mm long, bristly with a few long hairs outside, free, green, linear lanceolate and unequal. Numerous branched, more or less densely hirsuit with spreading hairs are found in the stem and the root system is tap root and branched [28]

In some African countries, another ethnopharmacological survey reports that *H. indicum* is believed to be useful in treating malaria, abdominal pain and dermatitis. The highest number of usages (22%) was reported for the treatment of malaria [29]. In Jamaica, the decoction of the entire plant is taken orally for treatment of intractable fever, ulcers, venereal diseases and sore throat and used externally in vaginal cavity to induce abortion in pregnant females and administered rectally to treat local sores in the rectum [30], while in Phillipines and Senegal, used orally as diuretic and for the treatment of kidney stone [30].

The infusion of the flower is taken orally by females for the treatment of menorrhagia [31]. In Rodrigues, the decoction of the entire plant is used externally for treating herpes and the paste of fresh plant is used externally for cleansing and dressing of wounds and ulcers. The sap of the stem is used orally by females for treating dysmenorrhea [31]. The hot water extract of the flower is taken orally by the females as an emmenagouge in small dose and abortive in large dose while a paste of fresh entire plant is used externally for treatment of head lice in the West Indies [32].

The leaf paste is applied externally to cure rheumatism in Rayal Seema in Andhra Pradesh, India. In Amazon, the paste of both leaf and root together is applied externally in scorpion stings, bug bites while the paste is recommended for treating sores and warts in Taiwan [33].

Extracts of several plants were used for their wound healing properties [34]. *Heliotrpium indicim* is an herb grown throughout Africa. This herb was used in Ivorian Pharmacopeia for it antipyretic; antiasthmatic effects. The plant is reported to be highly valued in the folklore medicine and is believed to be useful in treating malaria, abdominal pain, fever, dermatitis, venereal diseases, insect bites, menstrual disorder, urticaria, and sore throat. The plant decoction is considered as diuretic and remedy for the treatment of kidney stone. The leaf paste is applied externally to cure rheumatism and skin infections. The various tribes in India use the leaf paste over fresh cuts and wounds and claim for its promising activity [35]. A number of natural products from *H. indicum* such as volatile oil, Indicine-*N*-oxide, esters and terpenes have shown potent wound healing, antitumor and antileukemic activities [36].

	
Scientific classification Kingdom: Plantae Order: Lamiales Family: Verbenaceae Genus: *Vitex*. Species: *Vitex negundo*	**Scientific classification** Kingdom: Plantae Order: (unplaced) Family: Boraginaceae Genus: *Heliotropium* Species: *H. indicum*

AIM AND PLAN OF WORK

3. AIM AND PLAN OF WORK

Herbs are preferred because they do not produce any adverse effect with respect to their popularity and therapeutic utility. There is evidence of herbs having been used in the treatment of various diseases. Hence both *V. nugendo* and *H. indicum* were selected for the present investigation on *in vitro* anticancer activity.

3.1 PLAN OF WORK

- Collection and Authentication of ***Vitex negundo*** and ***Heliotropium indicum***
- Shade drying and Pulverization

PHYTOCHEMICAL STUDIES OF *STEPHANIA JAPONICA*

- Extraction of Plant Material
- Preliminary Phytochemical Screening of Extracts

Extraction of Plant Material

Successive solvent extraction of air dried leaves of *Vitex negundo and Heliotropium indicum* by using the solvents like

- Chloroform
- Ethanol
- Water

PHARMACOLOGICAL STUDIES

- Brine Shrimp Lethality Bioassay
- *In vitro* anticancer activity in human cancer cell lines

RESULTS & DISCUSSION

CONCLUSION

METHODOLOGY

4. MATERIALS AND METHODS

4.1 Plant Materials

4.2 Preparation of extracts of *Vitex negundo* and *Heliotropium indicum*

The powdered leaves (1kg) were sequentially extracted using chloroform, ethanol and aqueous solution in Soxhlet apparatus. After about forty siphons of each solvent extraction step, the materials were concentrated by evaporation. [30]

4.3 Preliminary phytochemical screening

Extracts of *Vitex negundo* and *Heliotropium indicum* were subjected to qualitative tests for the identification of various active constituents' viz. carbohydrate, glycoside, alkaloid, amino acids, flavanoids, fixed oil, tannins, gum and mucilage, phytosterols etc. The phytoconstituents were identified by chemical tests, which showed the presence of various constituents in the different extracts [33].

4.4 Brine Shrimp Lethality Bioassay:

The cytotoxic potential of extract of *V. nugendo* and *H. indicum* was determined by Brine shrimp lethality bioassay. Briefly, eggs of brine shrimp Artemia salina were hatched in a container filled with airbubbled artificial sea water which was prepared using 10 g of a commercial salt mixture (GEX Inc., Osaka, Japan) and 500 ml of distilled water. After 36-48 hours, the phototropic shrimps were collected and used for bioassay. To the vials containing different concentrations of extracts in sea water (1, 10, 25, 50 and 100 μg/ml), 25 shrimps were added and the vials were incubated at 25^{o}C and the surviving shrimps were counted after 24 hours. TheLC_{50} values of extracts greater than1000 μg/ml were considered inactive (non-toxic). Potassium dichromate was used as reference standard [37-39].

4. 5 *In vitro* Anticancer activity

4. 5. 1 Cell lines and culture conditions

HeLa (Human cervical cancer cell line) and PC3 (Human prostate cancer cell line) cell lines were procured from NCCS, Pune, India. Cells were grown in Minimum Essential Medium Eagle (Gibco, UK) supplemented with 10% heat inactivated fetal bovine serum (Gibco, UK), 29 µg/ml L-glutamine, and 40 µg/ml Gentamicin. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C.

4. 5. 2 Antiproliferative activity

The antiproliferative activity of plant extracts was measured using MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide) assay (Promega, USA). The assay detects the reduction of MTT by mitochondrial dehydrogenase to blue formazan product, which reflects the normal function of mitochondria and cell viability [38].

Exponentially growing cells were washed and seeded at 17000 cells/well (in 200 µl of growth medium) in 96 well microplates (Nunc, Denmark). After 24 h incubation, a partial monolayer was formed then the media was removed and 200 µl of the medium containing the plant extract (initially dissolved in DMSO) were added and re-incubated for 48 h. Then 100 µl of the medium were aspirated and 15 µl of the MTT solution were added to the remaining medium (100 µl) in each well. After 4 h contact with the MTT solution, blue crystals were formed. 100 µl of the stop solution were added and incubated further for 1h. Reduced MTT was assayed at 550 nm using a microplate reader (Das, Italy). Control groups received the same amount of DMSO (0.1%).Untreated cells were used as a negative control while, cells treated with vincristine sulfate were used as a positive control at the following concentrations 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 100 nM.

IC_{50} values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell line.

Stock solutions of the plant extract were dissolved in (DMSO) then diluted with the medium and sterilized using 0.2 μm membrane filters. The final dilution of extracts used for treating the cells contained not more than 0.1% DMSO. Data were reported as the average of three replicates. The antiproliferative effect of the tested extracts was determined by comparing the optical density of the treated cells against the optical density of the control (untreated cells).

4. 5. 3 DNA fragmentation assay

To determine the extracts induce apoptosis in SiHa cells, DNA fragmentation assay by agarose gel electrophoresis was performed [ ]. The cells (1×10^6) were seeded in 60 mm tissues culture dish treated with or without drug and incubated for 48 h. Cells were harvested by centrifugation and lysed in ice for 30 min by the addition of 20 μl lysis buffer contains 20 mM EDTA, 100 mM Tris (pH 8.0), and 0.8% (w/v) sodium lauryl sarcosine. The lysates was digested with RNase A (2 μl, 5 mg/ml) and proteinase K (20 μl, 10 mg/μl) at 37˚C for 1 h and 2 h, respectively. Total lysates were loaded onto 1.5% agarose gel stained with 0.5 μg/ml ethidium bromide and separated at 50 mV. DNA fragments were visualized by the Gel Doc 100 system (Bio-Rad; Hercules, CA).

4.6 Statistical Analysis

All experiments were repeated at least three times. At least quadruplicate cultures were scored for an experimental point. All values were expressed as mean ± S.E.M. The Student's one tail t-test was applied for statistical treatment of the results; $p < 0.05$ were considered as the statistically significant value.

RESULTS

5. RESULTS

The preliminary phytochemical screening of *V. nugendo* and *H. indicum* leaf extracts showed presence of steroids and sterols, triterpenoids, alkaloids, flavonoids, saponins, tannins and phenolic substances, gums and mucilages, carbohydrates, and proteins, respectively, in different extracts (Table 2).

5. 1 Brine Shrimp Lethality Bioassay

The result of cytotoxic potential of ethanol extract of *V. nugendo* and *H. indicum* in terms of mortality of brine shrimps (%) is presented in Figure 1. The degree of lethality was directly proportional to the concentration of the extracts. The percentage mortality of shrimps was recorded higher in case of *V. nugendo* LC_{50} were 50 and 25 µg/ml of chloroform and ethanol extract respectively (Figure 1) than that of *H. indicum* LC_{50} were 25 and 25 µg/ml of chloroform and ethanol extract respectively (Figure 2). Extract of *H. indicum* showed more lethality when compared with the reference control i.e., potassium dichromate (LC_{50} 32.77µg/ml). Highest mortality (100%) was observed at concentration 100 µg/ml of both the extracts. The aqueous extracts of both the plats didn't show any mortality in brine shrimp assay.

5.2 *In Vitro* Anticancer Activity

5.2.1 Antiproliferative activity

Human prostate cancer cell line (PC-3) and Human cervical cancer cell line (HeLa) were exposed to chloroform and ethanol extract of *V. nugendo* and *H. indicum* for 24 h and cytotoxicity was determined with the MTT assay. The Percentage cancer cell inhibition profiles were found to be concentration dependent (Figures 3-6). The maximum concentration used in the study was 500 mg/ml. HeLa cell lines, when

subjected to different concentrations of plant extracts displayed weak inhibition of 31.25%. It was observed from figures 3-6 that a gradual increase in percentage inhibition was observed in all the cases.

The extracts of *V.nugendo* exhibited significant cytotoxic activity against PC3 cell line with IC_{50} values of 70.23 mg/ml and 79.02 mg/ml for chloroform and ethanol fraction respectively (Figure 3), where good cytotoxicity were shown against HeLa cells with IC_{50} values of 48.13 mg/ml ad 59.87 mg/ml for chloroform and ethanol fraction respectively (Figure 5). Similarly, the extracts of *H. indicum* exhibited IC_{50} of 67.23 mg/ml and 72.23 mg/ml for chloroform and ethanol fraction respectively (Figure 4), where good cytotoxicity were shown against HeLa cell line with IC_{50} values of 58.43 mg/ml ad 64.23 mg/ml for chloroform and ethanol fraction respectively (Figure 6). Whereas, aqueous extracts of both the plants were found to be non toxic in both the cell lines.

5.2.1 DNA fragmentation assay

Induction of apoptosis on HeLa and PC3 cells by *V. nugendo* and *H. indicum* extracts was validated by DNA fragmentation analysis using gel electrophoresis technique. The DNA bands obtained from both extract-treated HeLa and PC3 produced ladder pattern as observed from Lane 2 to 7 (Figure 7). A ladder formation was used to indicate that the DNA has undergone fragmentation, and each fragment corresponded to a band in the ladder.

Table 1: Successive extraction of Chloroform, ethanol ad aqueous extracts of *Vitex negundo* and *Heliotropium indicum*

S.No.	Extracts	Percentage yield of extracts of *Vitex negundo* w/w	Percentage yield of extracts of *Heliotropium indicum* w/w
1.	Chloroform	4.23	2.12
2.	Ethanol	5.35	8.34
3.	Aqueous	13.8	9.15

Table 2: Phytochemical screening of Chloroform, ethanol ad aqueous extracts of *Vitex negundo* and *Heliotropium indicum*

Tests	*Vitex negundo*			*Heliotropium indicum*		
	CE	**EE**	**AE**	**CE**	**EE**	**AE**
Alkaloids	+	+	-	+	+	-
Carbohydrates	-	-	+	-	-	+
Glycosides	-	-	-	-	-	-
Gums and mucilages	-	-	+	-	-	+
Proteins and amino acids	-	-	-	-	+	-
Tannins and phenolic compounds	-	+	-	-	+	-
Steroids and sterols	+	+	-	+	-	-
Triterpenoids	+	-	-	+	-	
Saponins	-	-	+	-	+	+
Flavonoids	-	-	+	-	+	+

(+) indicates presence and **(-)** indicates absence of phytochemicals

CE – Chloroform extract, **EE** – Ethanol extract, **AE** – Aqueous extract

Table 3: Brine shrimp lethality bioassay of *Vitex negundo* and *Heliotropium indicum*

Conc. (µg/ml)	**LogC**	**% Mortality (*Vitex negundo)***			**% Mortality (*Heliotropium indicum)***		
		Chloroform extract	**Ethanol extract**	**Aqueous extract**	**Chloroform extract**	**Ethanol extract**	**Aqueous extract**
200	2.301	100	90	100	90	100	100
100	2.000	80	80	100	80	80	100
50	1.699	50	70	100	70	70	100
25	1.398	45	50	90	50	50	90
10	1.010	40	40	80	40	50	80
5	0.750	30	30	70	30	40	70
3	0.450	20	20	70	20	40	60
1	0.150	10	10	60	10	30	50

Figure 1: Brine shrimp lethality bioassay of *Vitex negudo*

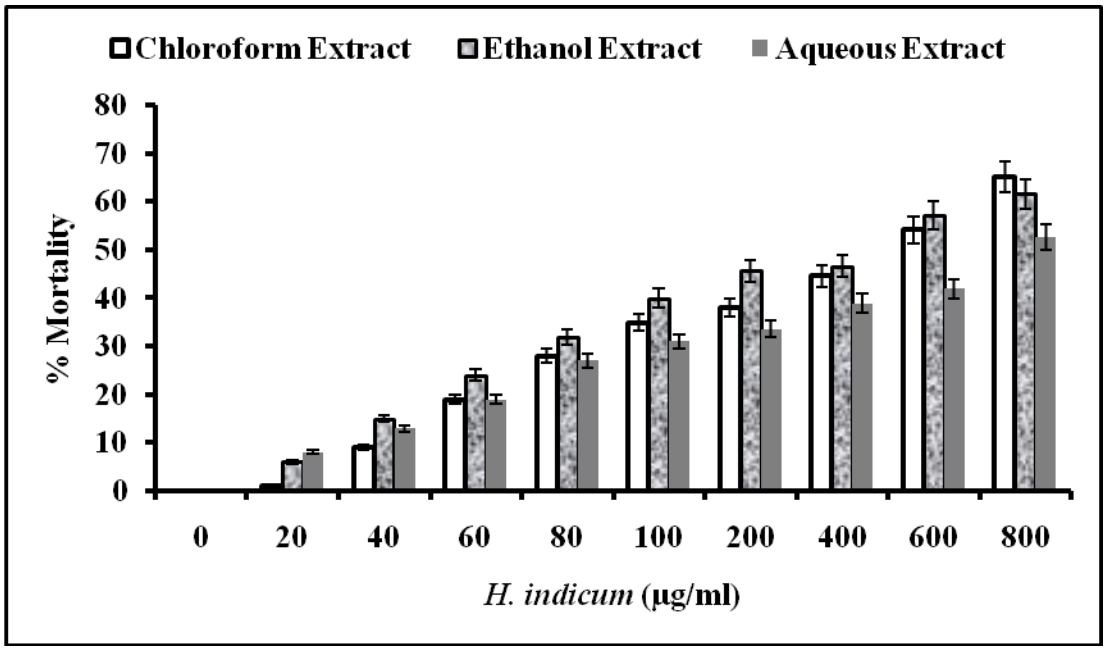


Figure 2: Brine shrimp lethality bioassay of *Heliotropium indicum*

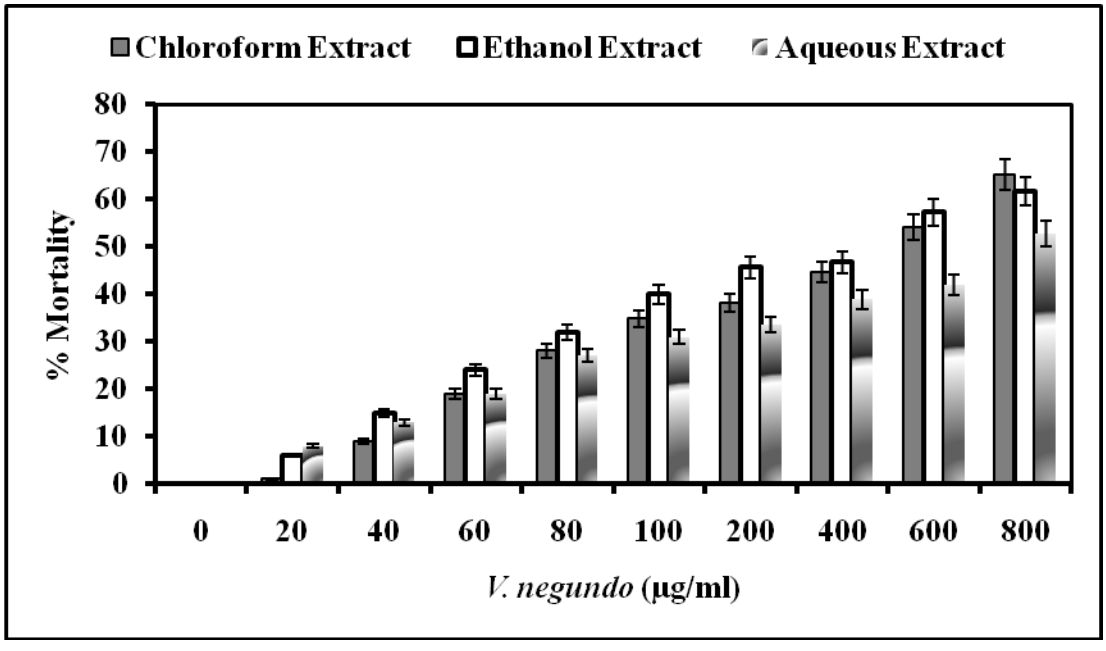


Figure 3: Antiproliferative activity of extracts of *Vitex negundo* in PC3 cell line

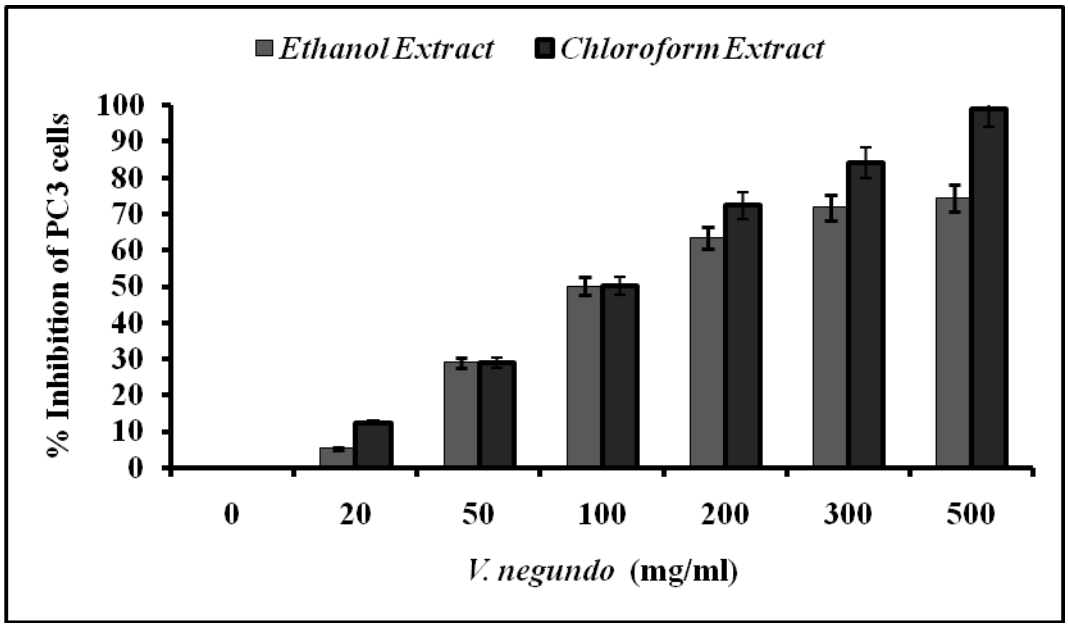


Figure 4: Antiproliferative activity of extracts of *Vitex negundo* in HeLa cell line

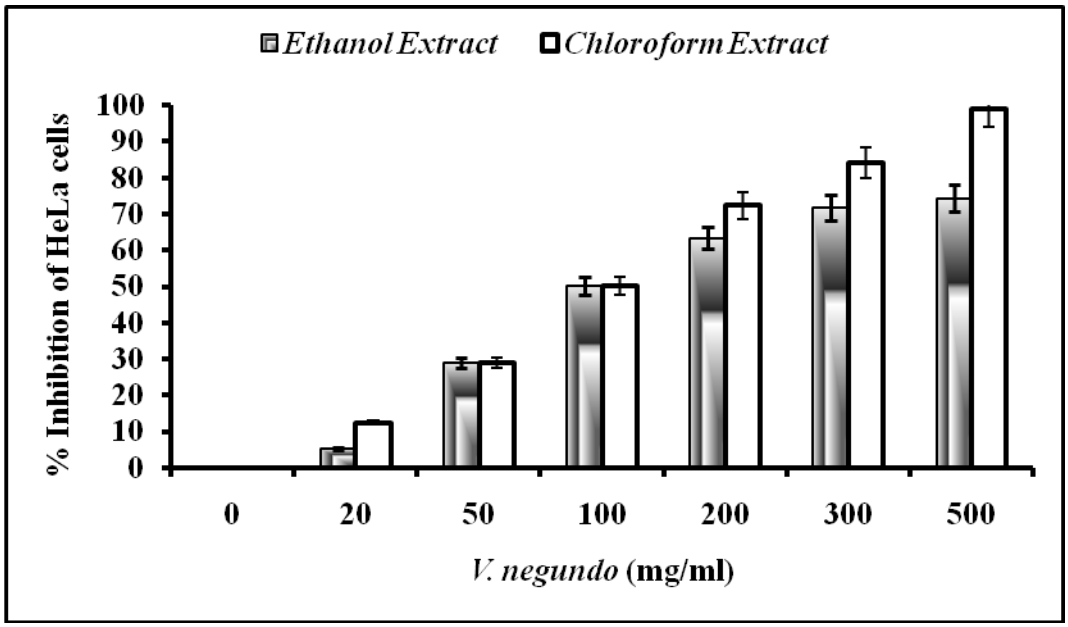


Figure 5: Antiproliferative activity of extracts of *Heliotropium indicum* in PC3 cell line

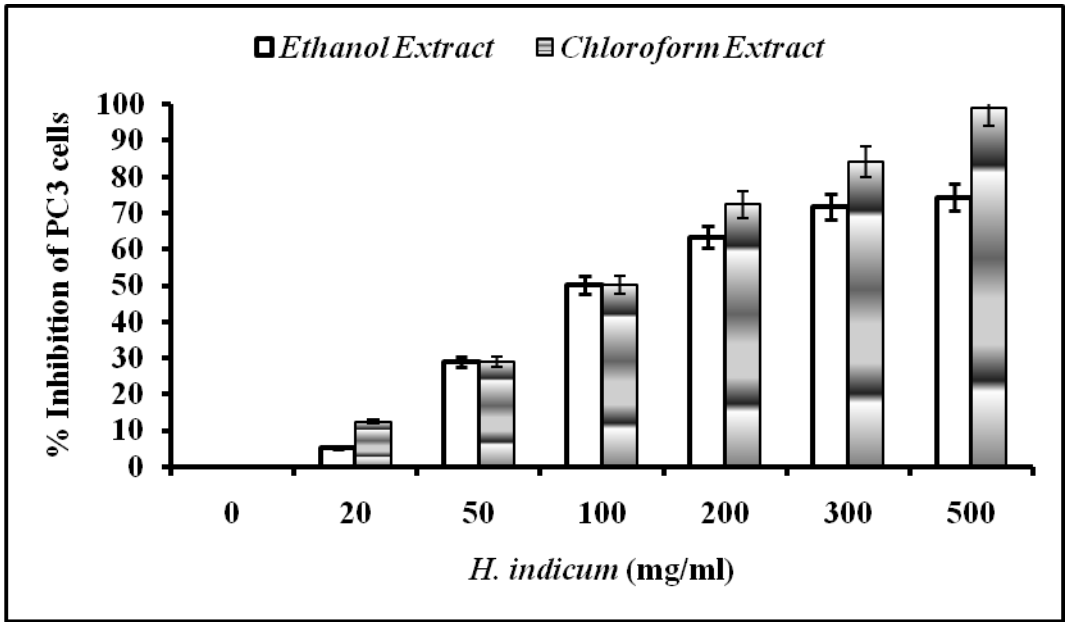
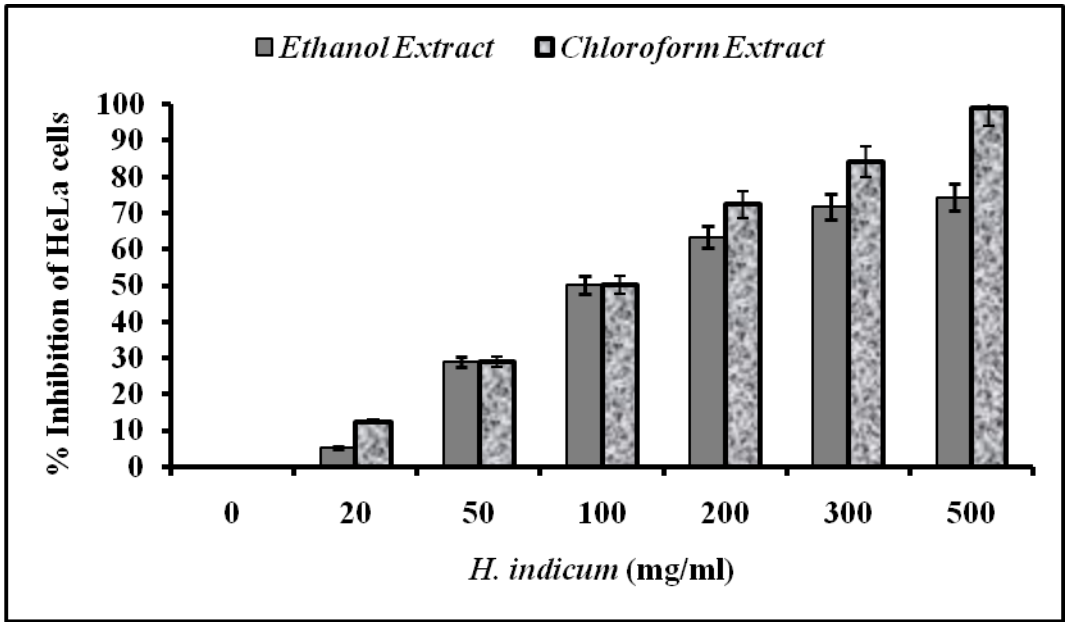
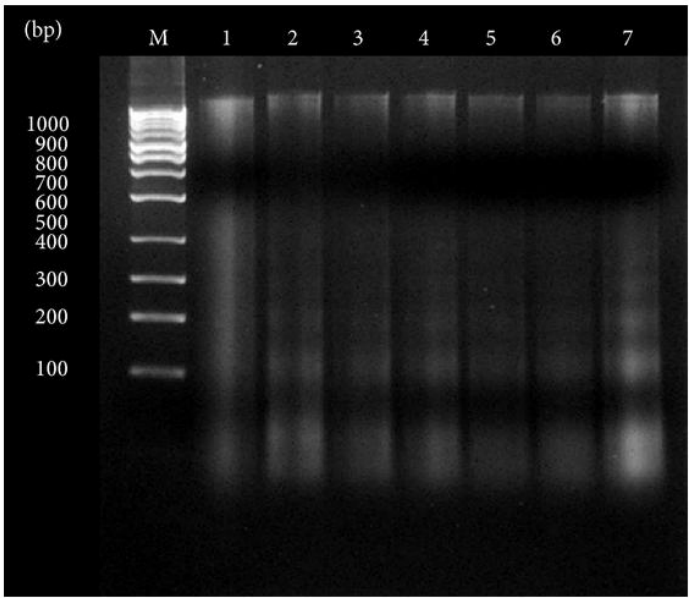
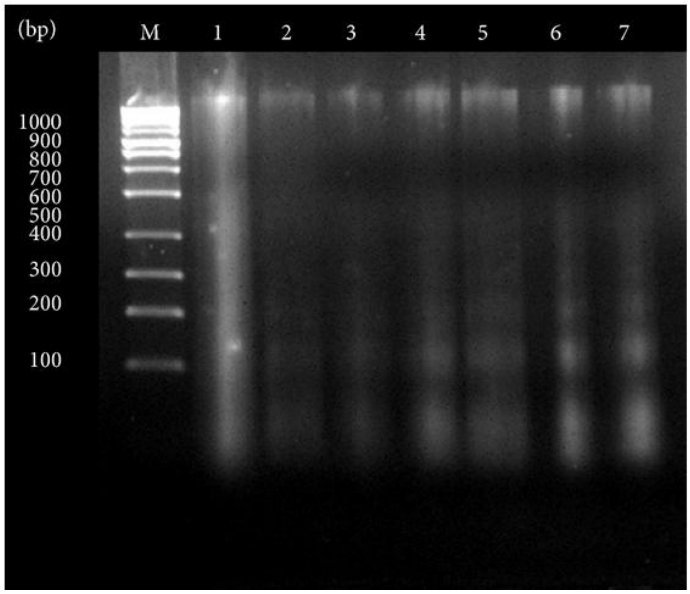


Figure 6: Antiproliferative activity of extracts of *Heliotropium indicum* in HeLa cell line





(a)



(b)

Figure 7: DNA band patterns of (a) HeLa and (b) PC3 cells treated with various concentrations of *Heliotropium indicum* and *Vitex negundo*. Lane 1: negative control; Lanes 2 to 3 were bands of cancer cells treated with 100 and 200 mg/mL extract of *Vitex negund.* Lanes 4 to 7with 100 and 200 mg/mL extract of *Heliotropium indicum.*

DISCUSSION

6. DISCUSSION

Ever since the existence of human being, plants have been exploited for several purposes including medicinal purposes. Plants are the primary source of biologically active phytochemicals present in conventional medicaments. Medicinal systems *viz*., Ayurveda, Unani and Sidda employ the use of these plants for treatment of diseases. Ethnobotanical studies highlight the relationships between various cultures and the traditional use of plants. Several ethnic groups all over the world employ a number of plant species for treatment of various ailments ranging from mild infections to fatal infections. Often, these studies are of importance and provide essential information for development of scientific research to justify the therapeutic potential of plants [40].

Brine shrimp lethality bioassay is an *in vivo* lethality assay that employs a simple zoologic organism as a convenient monitor for screening, discovering and monitoring various bioactivities of natural compounds. This test is very useful in determining various biological activities such as cytotoxic, phototoxic, pesticidal, trypanocidal, enzyme inhibition, and ion regulation activities. The assay can also be extrapolated for cell-line toxicity and antitumor activity. The method is rapid as it utilizes only 24 hours, inexpensive and needs no special equipment. It is even simple in that it does not require aseptic conditions to perform. The assay employs large number of organisms for validation and a relatively small amount of sample. This bioassay has been employed to determine cytotoxic activity of plant extracts [41-44]. In our study, the ethanol extract of *V. negundo* and *H. indicum* displayed cytotoxic activity as evidenced by the dose dependent mortality of brine shrimp larvae. Among extracts, higher cytotoxicity was observed in case of *H. indicum* extract than that of extract of *V. negundo*. High mortality

of shrimps caused by extract of *H. indicum* could be ascribed to the presence of high phenolic and flavonoid content. In another study [45] showed cytotoxic activity in terms of brine shrimp mortality of two compounds isolated from leaves of *V. negundo*. Crude ethanol extract and solvent fractions of bark of *V. negundo* were shown to exhibit marked cytotoxic effect in terms of mortality of brine shrimp larvae [45].

The reported results show that ethanolic extract of *H. indicum* has significant anticancer effect on prostate cancer (PC-3 cell line) compared to cervical cancer cell line (HeLa). The earlier work revealed that the ethanol extract of *H. indicum* leaves possess cytotoxic and anticancer properties [46].

Apoptosis is generally considered an energy-dependent process requiring active participation of many proteins and other cellular macromolecules. It is due to the fact that most of the intense genotoxic stimuli damage the proteins (or genes which are making those proteins) and other cellular macromolecules which may be required for apoptosis. The damage to proteins would result in their denaturation. This denaturation would confine the damaged DNA to the nuclear area giving a sharper outline to the nuclear boundary in necrotic cells. This sharpness in outline in necrotic cells may also be due to larger sized DNA, which does not diffuse as it does in apoptotic cells where DNA is as small as 180 bases.

Verification of the apoptotic activity was carried out based on the pattern of DNA bands produced from a gel electrophoresis. In apoptosis, cells are lysed gradually and systematically to produce membrane-bound apoptotic bodies, which was suggested to play a major role in suppressing inflammatory responses to other neighbouring cells. Apoptotic bodies or cells which underwent apoptosis produce a specific pattern of DNA

fragments with the multiples of 200 bp due to specific action of activated nucleases. These isolated fragments produced bands in a ladder pattern, in contrast with the smeared pattern produced from necrosis activity (Figure 7). Results obtained in this study in a way supported the various claims made by researches on the anticancer properties of these plant [10-15]. Further studies are being carried out to identify the active principle of the extract. Thus, it can be concluded that the strong antiproliferative activity of extract on cancer cells suggests its possible development as an anticancer agent. The mode of action of the extract was by the induction of apoptotic activity on cancer cells.

CONCLUSION

7. CONCLUSION

Natural products discovered from medicinal plants have played an important role in the treatment of cancer. The present study points to the potential anticancer activity of chloroform and ethanol extract of *V. negundo* and *H. indicum*. Further studies to characterize the active principles and elucidate the mechanism of the action of ethanol and chloroform extract are in progress.

Hence these plant extracts may have clinical and therapeutic proposition in the most life threaten disease like cancer and further studies are required to investigate these plant samples as antineoplastic agents. Therefore, it is anticipated that plants can provide potential bioactive compounds for the development of new 'leads' to combat cancer diseases.

REFERENCES

8. REFERENCES

1. Ernst E. The efficacy of herbal medicine – an overview. *Fund Clin Pharmacol*. 2005; 19: 405-409.
2. Rates SMK. Plants as source of drugs. *Toxicon*. 2001; 39: 603-613.
3. Balasubramanian A, Ramalingam K, Krishnan S, Christina AJM. Anti-inflammatory activity of *Morus indica* Linn. *Iran J Pharmacol Ther.* 2005; 4: 13-15.
4. Duraipandiyan V, Ayyanar M and Ignacimuthu S. Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India, *BMC Comp. Alter. Med*. 2006; 6: 35-41.
5. Mahishi P, Srinivasa BH, Shivanna MB. Medicinal plant wealth of local communities in some villages in Shimoga District of Karnataka. *J Ethnopharmacol*. 2005; 98: 307-312.
6. Gupta AK, Tandon N and Sharma M. Quality standards of Indian medicinal plants, Indian Council of Medical Research, New Delhi, India, 2005.
7. Hoyer DL, Kung HC, Smith BL. *Natl. Vital. Stat. Rep*. 2005; 53: 1-48.
8. Hartwell JL. Plants used against cancer: a survey. Lawrence, MA. Quarterman Publications, 1982, pp 438-39.
9. Itokawa H, Wang X, Lee K-H. Homoharringtonine and related compounds. In: Cragg GM, Kingston, DGI, New-man D, (eds). Anticancer agents from natural products. Boca Raton, Florida, Brunner-Routledge Psychology Press, Taylor & Francis Group, 2005, pp 47-70.
10. Cragg GM, Newman DJ, Snader KM. Natural products in drug discovery and development. *J Nat Prod*. 1997; 60: 52-60.

11. Cragg GM, Newman DJ. Plants as source of anticancer agents. *J Ethnopharmacol*. 2005; 100: 72-79.
12. IARC Website - www.dep.iarc.fr (updated 19.04.2014)
13. American Cancer Society, Facts and figures, 1999.
14. Ames BN, Gold LS and Willett WC. The causes and prevention of cancer. *Proc. Natl. Acad. Sci*. 1995; 92: 5258–5265.
15. Boik J. Cancer and Natural Medicine, Oregon Medical Press, Princeton, MN, 1995.
16. Kathiresan K, Boopathy NS and Kavitha S. Coastal vegetation – an underexplored source of anticancer drugs. *Nat. Prod. Rad.* 2006; 5: 115-119.
17. Steinmetz KA and Potter JD. Vegetables, fruits and cancer: Cancer Causes Control (Suppl.). *J. Epidemiology.*1991; 2: 325-357.
18. Bansod MS, Harle UN, Vitex negundo L: Phytochemical constituents, traditional uses and pharmacological properties: Comprehensive review. *Pharmacology online Newsletter*. 2009; 1: 286-302.
19. Kirtikar KR, Basu BD, Indian Medicinal plants. Dehradun Publishers Ltd, India.1994; 1: 830-832.
20. Khare CP, Encyclopedia of India Medicinal plants. Spinger Verlange Berline Heidelberge New York, 2004; 474-476.
21. Sharma PC, Yelne MB, Dennis TJ, Database of Medicinal plants used in Ayurveda. New Delhi, CISR 2005, 450.
22. Chaturvedi GN, Singh RH, *Indian J Med Res*. 1965; 53: 71.
23. Hansal R, Leuckert C, Rimpler H, Schaaf KD, *Phytochem*.1965; 4: 19.

24. Gupta RK and Tandon VR. *Vitex Negundo* Linn (VN) Leaf Extract as an Adjuvant Therapy to Standard Anti-Inflammatory Drugs. *Indian J Med Res*. 2006; 124: 447-450.

25. Telang RS, Chatterjee S and Varshneya C. Studies on Analgesic and Anti-Inflammatory Activities of *Vitex Negundo* Linn. *Indian J. Pharmacol*. 1999; 31: 363-366.

26. Aranda E, Hernandez MM, Heraso C, Villarreal ML, Arispuro IV. Biological activities of crude plant extracts from *Vitex trifolia* L. *J. Ethnopharmacol.* 1999, 67(1): 37-44.

27. Pandey DP, Singh JP, Roy R, Singh VP and Pandey VB: Constituents of *Heliotropium indicum*. *Orient J Chem.* 1996; 12(3): 321-322.

28. Mattocks AR: Minor alkaloids of *H. indicum*. *J Chem Soc.* 1967; 329.

29. Dolui AK, Debnath M and De B. Phytochemical and insecticidal screening of the leaf extract of *Heliotropium indicum* L. *Asian J. Chem*. 2010; 22: 5331-5334.

30. Srinivas K, Rao MEB and Rao SS. Anti-inflammatory activity of *Heliotropium indicum* Linn. and *Leucas aspera spreng*. in albino rats. *Indian J. Pharmacol*. 2000; 32: 37-38.

31. Reddy MS, Suresh RJ and Rajeswara Rao. Wound-healing effects of *Heliotropium indicum, Plumbago zeylanicum* and *Acalypha indica* in rats. *J. Ethnopharmacol*. 2002; 79: 249–251.

32. Wiart C. Medicinal Plants of the Asia-Pacific: Drugs for the Future? 2006, World Scientific Publishing Co. Pte. Ltd., Singapore.

33. Andhiwal CK, Has C and Varshney RP. Chemical and Pharmacological studies of *Heliotropium indicum*. *Indian Drugs.* 1985; 22(11): 567-569.

34. Mourin NA, Sharmin T, Chowdhury SR, Islam F, Rahman MS, Rashid MA. Evaluation of bioactivities of Heliotropium indicum, a medicinal plant of Bangladesh. *The pharma innov jour* 2013; 2 (2).

35. Rahman MA, Mia MA and Shahid IZ. Pharmacological and phytochemical screen activities of roots of *Heliotropium indicum* Linn. *Pharmacology online*. 2011; 1: 185-192.

36. Kugelman M, Liu WC, Axelrod M, Mc Bride TJ and Rao KV. Indicine-*N*-Oxide: The antitumor principle of *Heliotropium indicum*. *Lloydia*. 1976; 39(2/3): 125.

37. Takeya K, Quio ZS, Hirobe C, Itokawa H. Cytotoxic trichilintype limonoids from *Melia azedarach*. *Bioorganic and Med Chem*. 1996; 1355-1359.

38. Zhao G, Hui Y, Rupprecht JK, McLaughlin JL, Wood KV. Additional bioactive compounds and trilobacin, a novel highly cytotoxic acetogenin, from the bark of *Asimina triloba*. *J. Nat Prodt*. 1992; 55: 347-356.

39. Lau CS, Ho CY, Kim CF, Leung KN, Fung KP, Tse TF, Chan HL, Chow MS. Cytotoxic activities of Coriolus versicolor (Yunzhi) extract on human leukemia and lymphoma cells by induction of apoptosis. *Life Sci*. 2004; 75: 797–808.

40. Dayal R, Singh V. A comparative study of volatile constituent of Vitex negundo Leaves. *J Med Aromat Plant Sci.*2000; 22: 639-640

41. Dharmasiri MG, Jayakody JR, Galhena G, Liyanage SS, Ratnasooriya WD. Anti-inflammatory and analgesic activities of mature fresh leaves of *Vitex negundo*. *J Ethnopharmacol.* 2003; 87: 199-206.

42. Mayer B N, Ferrigni N R, Putam J E, Jacobsen L B, Nichols D E and Mclaughlin J L. Brine shrimp: a convenient bioassay for active plant constituents. *Plant Media.* 1982; 45: 31-34.

43. Goldstein A, Arnow L, Kalkan M and Summer M. Principles of drug action. 2nd Wiley Biomedical Health publication. 1974, pp.376-381.

44. Vander-Berghe Da, Vlietinck N. Screening methods for antibacterial and antiviral agents from higher plants. In: Dey PM, Harborne JB (eds). Methods in plant biochemistry, London: Academic Press, 1991.

45. Pranay Dogra, Study of Antibacterial and Anticancer Activity of Selected Trifoliate Plants. *Biofrontiers*. 2009; 1(2): 4-8.

46. Rajesh M Patel, Sahil K Patel, Cytotoxic activity of methanolic extract of *Artocarpus heterophyllus* against A549, Hela and MCF-7 cell lines. *J Appl Pharma Sci*. 2011; 01(07): 167-171.

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Committee for the Purpose of Control and Supervision of Experiments on Animals(CPCSEA)

Institutional Animal Ethics Committee (IAEC)

REG: NO: 887/PO/Re/S/2005/CPSCEA

CERTIFICATE

Title of the Project	:	*IN VITRO* ANTI-CANCER ACTIVITIES OF *VITEX NEGUNDO* AND *HELIOTROPIUM INDICUM* EXTRACTS AGAINST HUMAN CANCER CELL LINES .
Department	:	Pharmacology.
Proposal Number	:	JKKNCP/MP/OCT/03/2015-16
Approval date	:	18.01.2016
Animals	:	NA
No of Animals Sanctioned	:	NA

Dr. R. SAMBATH KUMAR
Chairman IAEC